Activation of TLR3 in Keratinocytes Increases Expression of Genes Involved in Formation of the Epidermis, Lipid Accumulation, and Epidermal Organelles

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Injury to the skin, and the subsequent release of noncoding double-stranded RNA (dsRNA) from necrotic keratinocytes, has been identified as an endogenous activator of Toll-like receptor 3 (TLR3). As changes in keratinocyte growth and differentiation follow injury, we hypothesized that TLR3 might trigger some elements of the barrier repair program in keratinocytes. dsRNA was observed to induce TLR3-dependent increases in human keratinocyte mRNA abundance for *ABCA12* (ATP-binding cassette, sub-family A, member 12), glucocerebrosidase, acid sphingomyelinase, and transglutaminase 1. Additionally, treatment with dsRNA resulted in increases in sphingomyelin and morphologic changes including increased epidermal lipid staining by Oil Red O and TLR3-dependent increases in lamellar bodies and keratohyalin granules. These observations show that dsRNA can stimulate some events in keratinocytes that are important for skin barrier repair and maintenance.

Journal of Investigative Dermatology (2013) **133,** 2031–2040; doi:10.1038/jid.2013.39; published online 28 February 2013

INTRODUCTION

Rapid recovery of epidermal barrier function following injury prevents water loss and opportunistic infection by infiltrating microbes. Barrier repair after injury involves trafficking of lamellar bodies (LBs) to the stratum corneum (SC), where they secrete their contents (Menon *et al.*, 1992), and activation of several other genes, including the ATP-binding cassette sub-family A, member 12 (ABCA12), an essential lipid transporter in the epidermis responsible for harlequin ichthyosis (Akiyama *et al.*, 2005), and lamellar ichthyosis type 2 (Lefévre *et al.*, 2003), and lipid metabolism enzymes, glucocerebrosidase (GBA) (Holleran *et al.*, 1994) and acid sphingomyelinase (SMPD1) (Jensen *et al.*, 1999). Cholesterol (Menon *et al.*, 1985a), free fatty acid (Mao-Qiang *et al.*, 1993; Ottey *et al.*, 1995), and ceramide synthesis all also increase following skin barrier disruption (Holleran *et al.*, 1991a, 1995) and are essential for barrier repair. The mechanisms that regulate the complex events that comprise barrier repair are incompletely defined, though a calcium gradient in the epidermis plays an important role (Lee *et al.*, 1992; Menon *et al.*, 1992a). In this study we sought to test the hypothesis that double-stranded RNA (dsRNA), recently discovered to be an endogenous product produced by epidermal injury following trauma or excess UVB exposure (Lai *et al.*, 2009; Bernard *et al.*, 2012), might serve as a trigger for expression of genes important to the epidermal barrier repair process.

dsRNA recognition can occur by several mechanisms including binding to Toll-like receptor 3 (TLR3). TLR3 signaling has largely been described as a recognition and response system to combat viral infections (Kawai and Akira, 2008; Dunleavy *et al.*, 2010). Patients who have mutations in TLR3 (Zhang *et al.*, 2007), UNC-93B, an endoplasmic reticulum membrane protein important for its trafficking to the endosome, (Casrouge *et al.*, 2006), or TRIF (Toll/interleukin-1 receptor (TIR) domain-containing adapter-inducing IFN-β, a key adaptor signaling molecule for TLR3 signaling (Sancho-Shimizu *et al.*, 2011), are more susceptible to herpes simplex virus encephalitis. Thus, the importance of TLR3 in sensing viruses is not disputed, although more recently it has been shown to have an expanded role in a number of epithelial tissues. For example, inflammatory cytokines are induced by RNA released during necrosis in the gut (Cavassani *et al.*, 2008) or damage to the skin (Lai *et al.*, 2009; Bernard *et al.*, 2012). In addition, recent publications have also demonstrated

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Abbreviations: 3D, three dimensional; ABCA12, ATP-binding cassette, sub-family A, member 12; AD, atopic dermatitis; dsRNA, double-stranded RNA; FLG, filaggrin; GBA, glucocerebrosidase; KHG, keratohyalin granule; LB, lamellar body; LOR, loricrin; NHEK, normal human epidermal keratinocyte; Poly (I:C), polyinosinic acid:polycytidylic acid; SC, stratum corneum; SMPD1, acid sphingomyelinase; TGM1, transglutaminase 1; TLR3, Toll-like receptor 3; TNF, tumor necrosis factor

Received 3 August 2012; revised 7 December 2012; accepted 2 January 2013; accepted article preview online 25 January 2013; published online 28 February 2013

that TLR3 is important for wound healing, as *Tlr3*$^{-/-}$ mice have a slightly delayed wound healing phenotype (Lin *et al.*, 2011), whereas Polyinosinic acid:Polycytidylic acid (Poly(I:C)), a dsRNA analog and ligand for TLR3, can promote wound healing in mice (Lin *et al.*, 2012).

These recent findings suggest that TLR3 may have multiple functions in the skin and may signal the start of barrier repair processes in addition to its role in viral defense. We show herein that TLR3 activation increased expression of genes critical to barrier formation, increased the appearance of epidermal lipids, and increased LBs and keratohyalin granules (KHGs), important elements of the epithelial barrier repair response. These findings therefore identify TLR3 as a potential regulator of epidermal regeneration following injury.

RESULTS

Gene expression profiling of lipid metabolism and lipid transporter pathways

To identify gene expression pathways in addition to the known inflammatory response associated with TLR3 activation of keratinocytes, we examined the transcriptome of normal human epidermal keratinocytes (NHEKs) 24 hours after exposure to the dsRNA Poly(I:C). In response to Poly(I:C), a total of 5,542 differentially regulated genes changed by at least 2-fold (2,773 upregulated and 2,769 downregulated; Significance Analysis of Microarrays (SAM): triplicate; false discovery rate $<0.01\%$; delta value $=1.397$; Supplementary Figure S1a online). These genes were further analyzed using the Database for Annotation, Visualization and Integrated Discovery (DAVID) (Huang *et al.*, 2009a, b). This analysis suggested that Poly(I:C) affected a number of pathways involved in lipid metabolism and transport. Specifically, changes were observed in the expression of genes in glycosphingolipid biosynthesis, ABC transporters, sphingolipid metabolism, and other lipid biosynthesis/metabolism and inflammatory pathways (Figure 1a). Several specific genes identified by this approach are known to play a role in maintaining or forming the skin barrier, such as ABCA12 (3.74-fold), GBA (2.02-fold), SMPD1 (2.04-fold), transglutaminase 1 (TGM1; 2.40-fold), as well as tumor necrosis factor (TNF; 5.31-fold), IL-6 (27.31-fold), and TLR3 (14.58-fold). Involucrin, loricrin (LOR), keratin 1 (KRT1), keratin 14 (KRT14), and filaggrin (FLG), markers of epidermal differentiation, were not increased significantly (Figure 1b). The results of the microarray showed that genes involved in synthesis of ceramides (UGCG, SPTLC1, SPTLC2), free fatty acids (ACACA and FASN), and cholesterol (FDFT1, HMCCR, and HMGSC1) were not significantly altered other than acetyl CoA carboxylase (ACACA; 0.33-fold).

Poly(I:C) enhances transcript abundance of genes involved in skin barrier formation

To validate the results of the gene expression profile of Poly(I:C)-treated NHEKs, we measured a number of genes by real-time PCR. mRNA of ABCA12, GBA, SMPD1, and TGM1 were significantly upregulated following treatment with Poly(I:C) (Figure 2a and Table 1). As expected, traditional inflammatory markers TNF, IL-6, and TLR3 were also upregulated (Figure 2b and Table 1). Consistent with microarray data, this treatment did not induce expression of involucrin, KRT1, LOR, FLG, or KRT14 (Figure 2c and Table 1). Interestingly, Poly(I:C) treatment significantly induced expression of mRNA for ceramide synthesis enzymes, including serine palmitoyltransferase (SPTLC1 and SPTLC2) and glucosylceramide synthase (UGCG) (Figure 1b and Table 1). Ligands for TLRs 2, 7, 8, and 9 did not significantly alter expression of these barrier repair genes (Supplementary Figure S2a and b online). A dose-dependent increase in the mRNA levels of ABCA12, GBA, SMPD1, TNF, and IL-6 was observed following treatment with Poly(I:C), with maximal expression seen after treatment with 0.5 to 1 $\mu g\,ml^{-1}$ Poly(I:C) (Figure 2d). Increases in transcript abundance for ABCA12, GBA, and SMPD1 were not seen until 24 hours after treatment, whereas TNF expression increased more rapidly and was maximal at 1 hour (Figure 2e). However, the latter effect on gene expression was not because of induction of these cytokines as treatment of NHEKs with TNF or IL-6 had no significant effect on the induction of ABCA12, GBA, or SMPD1 mRNA levels (Figure 2e).

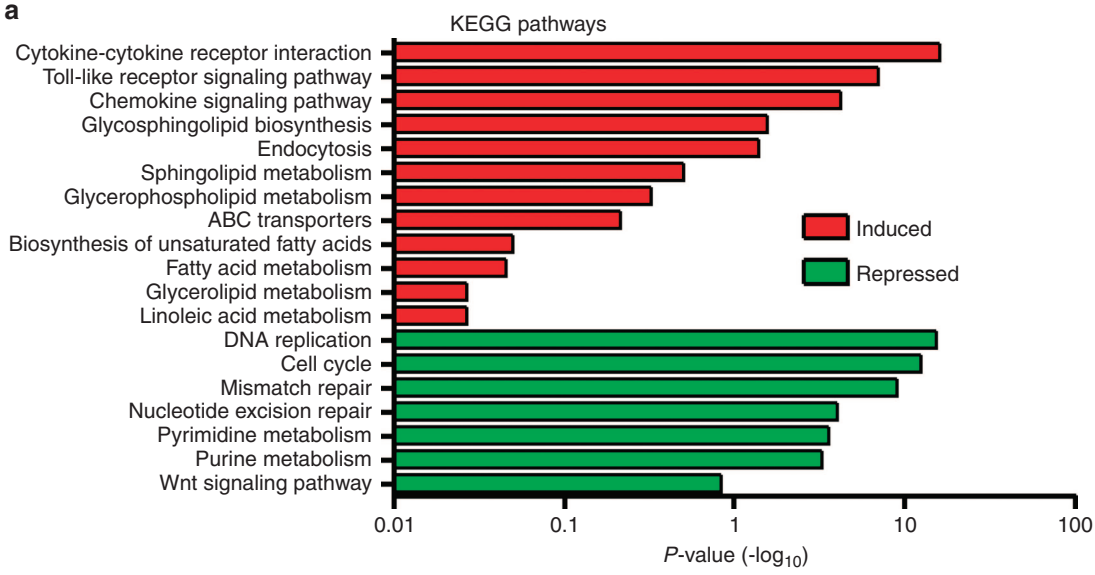
TLR3 activation is required for dsRNA-induced changes in gene expression

To determine if the increases in ABCA12, GBA, SMPD1, and TGM1 mRNA after Poly(I:C) treatment were dependent on TLR3 activation, we used small interfering RNA to knockdown TLR3 in NHEKs. When TLR3 was significantly decreased in keratinocytes, Poly(I:C) failed to induce a significant increase in mRNA for the barrier repair genes ABCA12, GBA, SMPD1, TGM1, and TNF (Figure 3a). As TLR3 signaling is dependent on proper acidification and maturation of endosomes (Matsushima *et al.*, 2004), we used Bafilomycin A1 (BafA1), a specific inhibitor of the V-type ATPase required for acidification of endosomes and lysosomes, to inhibit TLR3 signaling. BafA1 blocked Poly(I:C)-induced increases in ABCA12, GBA, and SMPD1 mRNA as well as increases in mRNA of the inflammatory cytokines TNF and IL-6 (Figure 3b). Similar effects on gene expression were seen when TRIF, a key signaling molecule downstream of TLR3, was knocked down (Supplementary Figure S4 online). Unlike silencing of TLR3 or TRIF, knocking down MAVS, a key signaling molecule for RIG-I-like receptors that recognizes cytosolic dsRNA, had no significant effect on Poly(I:C)-induced expression of ABCA12, GBA, SMPD1, and TGM1 (Supplementary Figure S5 online). Although TLR3 activation was important for Poly(I:C)-induced increases in UGCG mRNA, alterations in mRNA for several lipid synthesis genes was largely independent of TLR3 (Figure 3c).

Activation of TLR3 alters epidermal lipid content

To determine whether increases in ABCA12, GBA, and SMPD1 transcripts were paralleled by changes in epidermal lipid composition, we first stained for the presence of lipid in NHEKs grown in differentiating conditions. A large increase in Oil Red O-staining bodies was seen when NHEKs were exposed to Poly(I:C) for 72 hours (Figure 4a). A significantly higher expression of ABCA12 and GBA was also seen upon Poly(I:C) treatment under these conditions (Supplementary

a



b

Gene	Control			+ 1 μg ml^{-1} Poly(I:C)		
IL-6	0.95	0.92	1.13	16.45	21.09	35.75
TLR3	0.96	1.00	1.04	13.14	14.80	15.80
TNF	1.05	0.88	1.07	4.79	5.43	5.70
ABCA12	0.88	1.03	1.09	3.03	3.46	4.73
TGM1	1.07	1.02	0.91	2.31	2.50	2.38
SMPD1	1.02	0.91	1.07	1.93	1.98	2.23
GBA	0.98	0.97	1.05	1.95	1.97	2.16
MAVS	1.04	0.97	1.00	1.82	1.63	1.63
HMGCS1	0.91	1.06	1.03	1.22	1.65	1.93
LOR	1.26	0.84	0.90	2.28	1.37	0.95
SPTLC1	0.98	1.04	0.99	1.17	1.20	1.19
HMGCR	0.96	1.03	1.01	0.93	1.05	1.13
KRT14	1.08	1.00	0.93	1.07	1.08	0.91
FDFT1	1.04	0.93	1.03	0.85	0.80	0.89
FLG	0.92	0.99	1.10	0.90	0.79	0.82
SPTLC2	1.00	1.03	0.97	0.77	0.82	0.84
FASN	1.04	0.96	1.00	0.75	0.60	0.66
UGCG	0.95	1.02	1.03	0.58	0.72	0.71
IVL	0.92	1.07	1.01	0.48	0.65	0.66
KRT1	1.03	1.07	0.90	0.48	0.70	0.37
ACACA	0.89	1.06	1.05	0.30	0.34	0.35

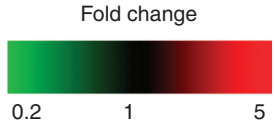


Figure 1. Gene expression profiling of normal human epidermal keratinocytes (NHEKs) identifies upregulation of genes involved in lipid biosynthesis, metabolism, and transporter pathways following treatment with double-stranded RNA (dsRNA). (**a**) Significantly changed genes analyzed using the Database for Annotation, Visualization and Integrated Discovery (DAVID) to identify significant pathways (EASE = 1.0). (**b**) Genes involved in skin barrier formation and dsRNA recognition. KEGG, Kyoto Encyclopedia of Genes and Genomes; Poly(I:C), polyinosinic acid:polycytidylic acid.

Figure S3a and b online). Lipids were then quantified by measuring the amount of Oil Red O dye that was retained after staining and normalizing this to total protein. Poly(I:C) treatment induced significant increases in lipids stained by Oil Red O at days 1–3 (Figure 4b). Next, three-dimensional (3D) skin constructs were exposed to Poly(I:C) to determine the response of stratified and differentiated keratinocytes that model the epidermis but are not influenced by the presence of resident or recruited leukocytes that would be present *in vivo*. In these 3D skin constructs, the SC of Poly(I:C)-treated

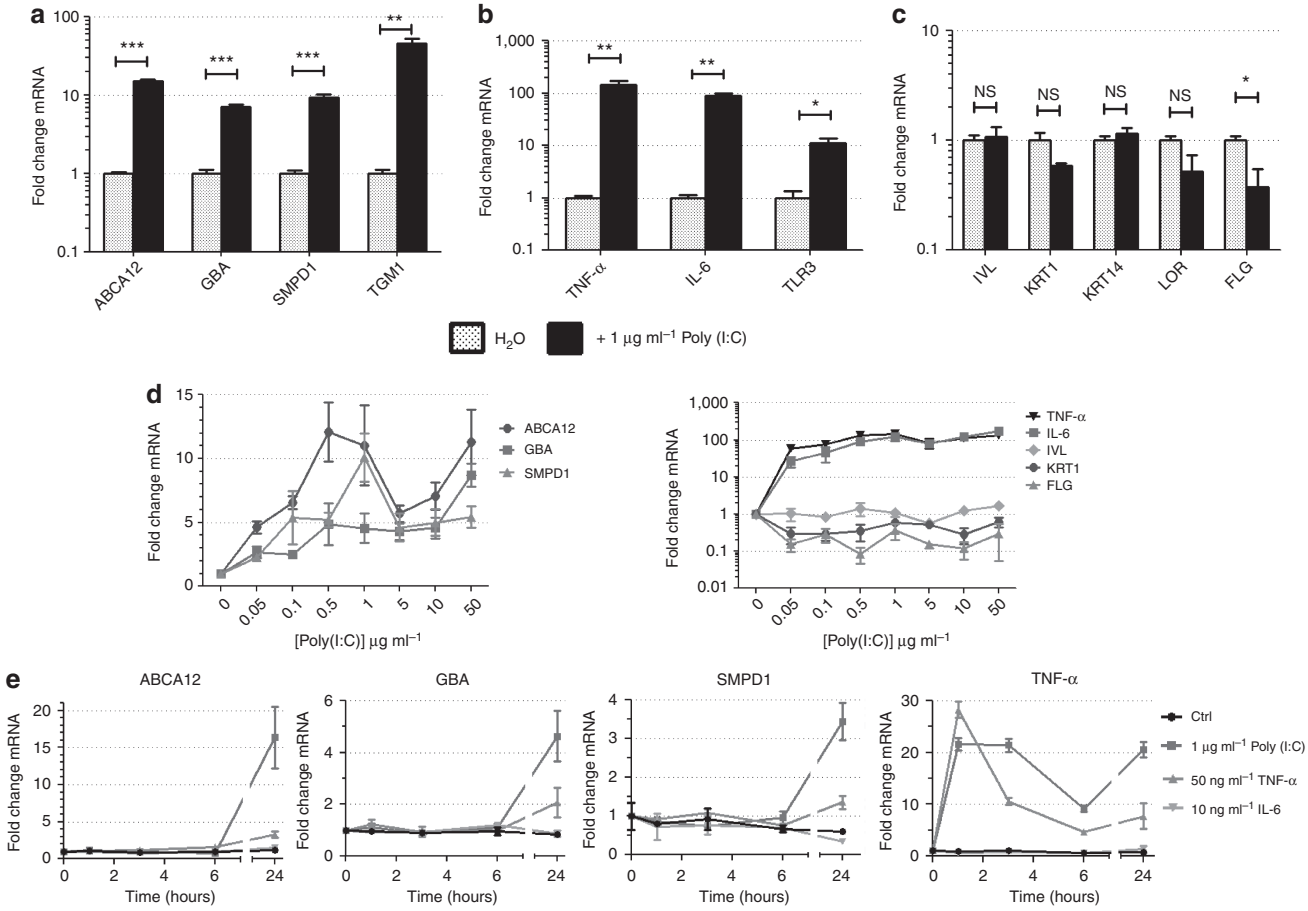


Figure 2. Polyinosinic acid:polycytidylic acid (Poly (I:C)) enhances transcript abundance of genes involved in skin barrier formation. Normal human epidermal keratinocyte (NHEKs) were cultured in the presence of 1 μg ml^{-1} Poly(I:C) for 24 hours. Real-time PCR was used to quantify mRNA levels and fold change values are calculated relative and normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression for a number of (**a**) barrier genes, (**b**) inflammatory cytokines, and (**c**) keratinocyte differentiation markers. (**d**) NHEKs were cultured with various doses of Poly(I:C) for 24 hours. (**e**) NHEKs were incubated with 1 μg ml^{-1} Poly(I:C), 50 ng ml^{-1} tumor necrosis factor (TNF), or 10 ng ml^{-1} IL-6 for 0, 1, 3, 6, or 24 hours. Data are mean ± SEM, $n=3$, and are representative of at least three independent experiments. *$P<0.05$; **$P<0.01$; ***$P<0.001$. Two-tailed *t*-test.

samples stained more strongly for Oil Red O compared with control samples (Figure 4c).

To measure the response of specific lipid components produced by cultured keratinocytes, total lipids were isolated from NHEKs after Poly(I:C) treatment and resolved using high-performance thin-layer chromatography. Sphingomyelin was significantly increased following Poly(I:C) treatment and this increase was blocked following small interfering RNA silencing of TLR3 (Figure 4d). Glucosylceramide levels were significantly decreased after Poly(I:C) treatment, although this change was independent of TLR3 (Figure 4e). Ceramides increased following Poly(I:C) treatment, although this increase was not abolished by knockdown of TLR3 (Figure 4f). Cholesterol levels were not significantly altered after Poly(I:C) treatment in either control or TLR3 knockdown keratinocytes (Figure 4g).

TLR3 activation increases the quantity of LBs and KHGs in the epidermis

As we observed increases in the staining of lipids following exposure to dsRNA, we next sought to determine if other morphological changes in keratinocytes could be observed by electron microscopy. To assess this, we quantitated the number of LBs and KHGs in the upper stratum granulosum in 3D epidermal skin constructs. Skin constructs treated with Poly(I:C) had significantly more LBs and KHGs (Figure 5a and b). The observed increases in LBs and KHGs were dependent on TLR3, as skin constructs generated from TLR3-knockdown NHEKs failed to exhibit significant increases in LBs and KHGs when exposed to Poly(I:C) (Figure 5c).

DISCUSSION

TLR activation is classically considered to result in proinflammatory responses. In this study, we demonstrate that TLR3 activation of keratinocytes also leads to changes in expression of some genes in keratinocytes that are associated with epidermal structure. An increase in transcript abundance of ABCA12, GBA, SMPD1, and TGM1 occurred in a TLR3-dependent manner. This response was followed by increases in epidermal lipid accumulation as well as increases in the abundance of LBs and KHGs in epidermal equivalents. These observations are consistent with recent observations that

Table 1. Poly(I:C)-induced gene expression changes

Gene name	Fold change (real-time PCR)	± SD	*t*-Test	Fold change (microarray)	SAM
ABCA12	22.68	5.551	**	3.74	+
GBA	10.84	1.243	***	2.03	+
SMPD1	9.48	1.143	***	2.05	+
TGM1	30.65	4.231	***	2.40	+
TNF	23.07	0.564	***	5.31	+
IL-6	41.99	2.480	***	27.31	+
TLR3	69.66	9.100	***	14.58	+
MAVS	1.40	0.309	NS	1.70	–
KRT1	1.33	1.727	NS	0.52	–
KRT14	0.74	0.012	***	1.02	–
IVL	0.79	0.064	NS	0.60	–
LOR	1.43	1.216	NS	1.53	–
FLG	0.83	1.224	NS	0.84	–
SPTLC1	1.36	0.071	**	1.19	–
SPTLC2	3.72	0.834	**	0.81	–
UGCG	2.61	0.174	***	0.67	–
ACACA	0.37	0.079	**	0.33	+
FASN	0.48	0.113	*	0.67	–
HMGCR	1.24	0.057	*	1.04	–
HMGCS1	1.07	0.143	NS	1.60	–
FDFT1	0.71	0.096	*	0.85	–

Abbreviations: NS, nonsignificant; Poly(I:C), polyinosinic acid:polycytidylic acid; SAM, Significance Analysis of Microarrays.
$*P<0.05$, $**P<0.01$, and $***P<0.001$. Student's *t*-test.
Data are mean of triplicate samples and representative of at least three independent experiments for real-time PCR. Data are mean of triplicate samples and analyzed for significance with SAM (fold change >2, false discovery rate (FDR) <0.01%; delta value = 1.397).
Data in table represent real-time PCR and microarray fold change data from normal human epidermal keratinocyte (NHEKs) treated with 1 μg ml^{-1} Poly(I:C) versus control, water-treated NHEKs.

dsRNA is released by damaged cells and can serve as a damage-associated molecular pattern. Thus, we now show that skin epithelial cells initiate some of the events associated with barrier repair after recognition of dsRNA.

The protective properties of the skin barrier reside in the SC and are heavily dependent on the lipid-rich lamellar membranes surrounding differentiated keratinocytes (Holleran *et al.*, 1991b; Feingold, 2007; Feingold and Denda, 2012). Previous studies have characterized the barrier repair response, delineating the increase in epidermal lipid synthesis and metabolism in the skin (Menon *et al.*, 1985a; Feingold, 1991; Holleran *et al.*, 1991a,b) and secretion of LBs following barrier disruption (Menon *et al.*, 1992b). Because our current observations show TLR3 activation is accompanied by increases in mRNA-encoding genes involved in epidermal formation, accumulation of epidermal lipids, and formation of epidermal organelles, we provide evidence that TLR3 may be a previously unknown mechanism by which keratinocytes detect epidermal injury and initiate some of the steps involved in formation of a functional skin barrier. However, as mice lacking TLR3 appear to develop normally, this recognition system is not critical to normal development. Furthermore, although many Poly(I:C)-induced changes in lipid composition and quantity were observed, not all of these changes were TLR3 dependent. It is important to keep in mind that we did not observe a global upregulation in lipid or differentiation markers. In contrast, we observed that dsRNA can induce TLR3-dependent changes only in specific elements involved in the process of repair. How these responses combine into the complete barrier repair program remain to be determined.

A number of receptors are known to recognize and respond to dsRNA (Yu and Levine, 2011), making it important to determine whether TLR3 activation was required for the gene expression changes seen in response to dsRNA. Using both RNA silencing and pharmacological inhibition, we demonstrated that increases in skin barrier genes in response to Poly(I:C) were dependent on TLR3. As the Poly(I:C)-induced changes in mRNA of these barrier genes were almost completely abrogated when TLR3 activation was silenced, our data suggest that TLR3 activation is required for the observed changes. These data do not rule out the contribution of cytoplasmic sensors of dsRNA that exist in the cell, including RIG-I, MDA5, PKR, and NLRP3, although the failure of MAVS knockdown to partially inhibit the Poly(I:C) response argues against a role for cytoplasmic RNA recognition. Thus, the relevance of these sensors in keratinocytes is yet to be clearly defined.

As changes in gene expression are not seen until 24 hours after Poly(I:C) treatment, it is possible that the change in transcription of ABCA12, GBA, SMPD1, and TGM1 is not a direct downstream transcriptional event of TLR3 activation, but rather an autocrine or paracrine effect dependent on synthesis of intermediate genes. TNF and IL-6 are produced following TLR3 activation and have been shown to improve the skin barrier (Jensen *et al.*, 1999; Wang *et al.*, 2004). Therefore, we also examined the direct effects of these cytokines on induction of the barrier genes of interest. As no changes in gene expression of ABCA12, GBA, or SMPD1 were observed with TNF or IL-6 treatment of keratinocytes under conditions similar to those where Poly(I:C) did induce these cytokines, it is unlikely that these cytokines acting alone are responsible for the observed effects. Future work will seek to better understand the factors responsible for transcriptional regulation of ABCA12, GBA, SMPD1, and TGM1 by TLR3, with specific interest in determining if these genes represent an immediate canonical response to TLR3 or are activated in response to stimulation of intermediate factors that may function in an autocrine manner.

Poly(I:C) recognition by TLR3 in keratinocytes appears to be functionally relevant. Sphingolipids, such as ceramides and its precursors sphingomyelin and glucosylceramides, are essential for the formation and maintenance of the skin barrier (Holleran *et al.*, 1991a). Poly(I:C)-treated keratinocytes displayed a rapid appearance of lipid-containing vesicles and an increase in ceramides. More intense lipid staining in the SC was also observed in 3D skin constructs treated with

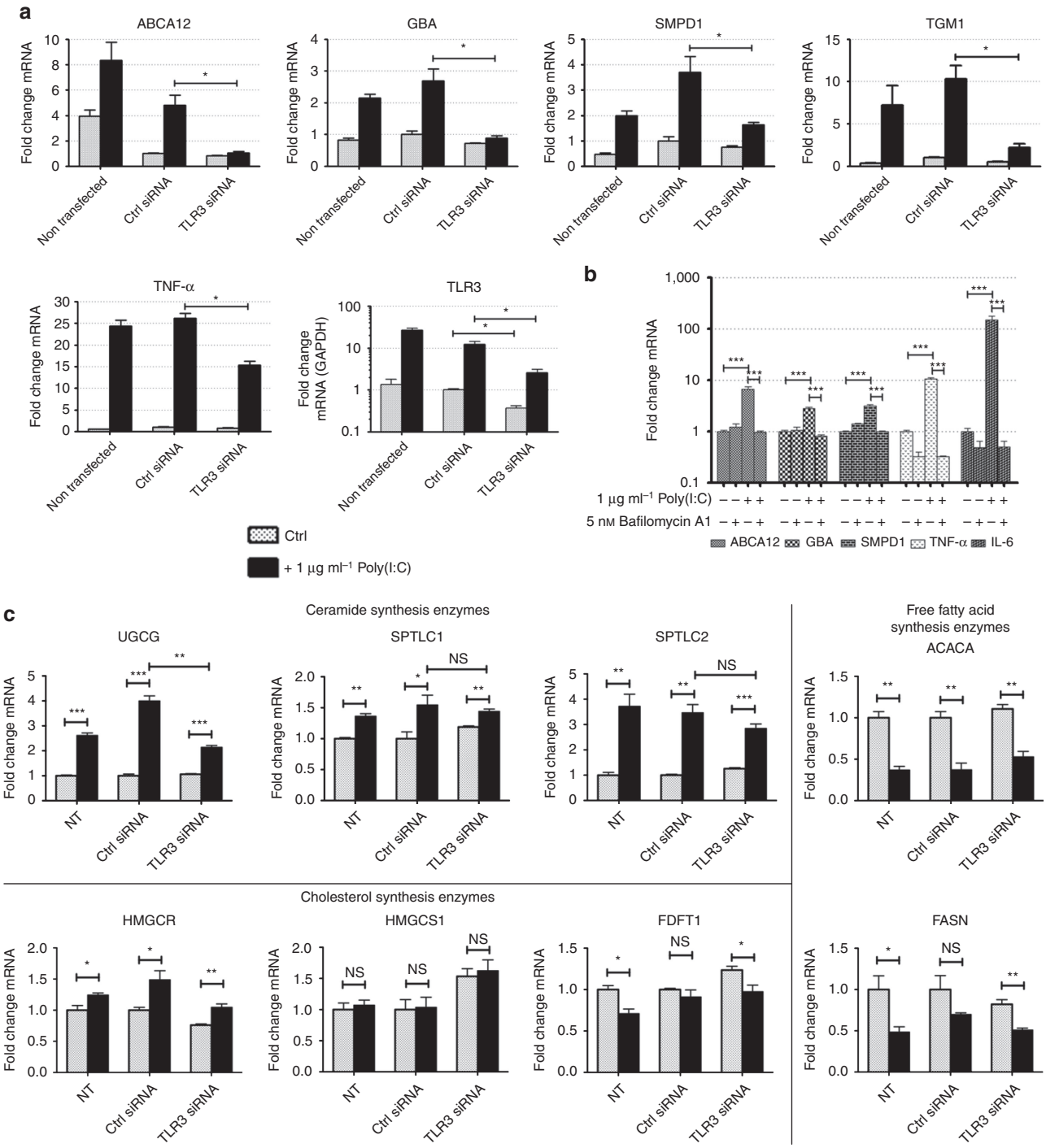


Figure 3. Toll-like receptor 3 (TLR3) activation is required for double-stranded RNA (dsRNA)-induced changes in gene expression. (**a**) TLR3 was silenced in normal human epidermal keratinocytes (NHEKs) for 48 hours before treatment with 1 μg ml^{-1} polyinosinic acid:polycytidylic acid (Poly(I:C)) for 24 hours. Real-time PCR was used to quantify mRNA levels and fold change values are calculated relative to and normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression. Two-tailed *t*-test. (**b**) NHEKs were treated with 5 nM Bafilomycin A1 for 1 hour before treatment with 1 μg ml^{-1} Poly(I:C) for 24 hours. One-way analysis of variance (ANOVA). (**c**) TLR3 was silenced in NHEKs for 48 hours before treatment with 1 μg ml^{-1} Poly(I:C) for 24 hours. Real-time PCR was used to quantify mRNA levels and fold change values are calculated relative to and normalized to GAPDH expression. *$P<0.05$; **$P<0.01$; ***$P<0.001$. NS, nonsignificant. Two-tailed *t*-test. Data are mean ± SEM, $n=3$, and are representative of at least three independent experiments.

Poly(I:C). In addition, sphingomyelin levels were increased by Poly(I:C) treatment in a TLR3-dependent manner. In contrast, levels of glucosylceramides decreased following Poly(I:C) treatment, but not in a TLR3-dependent manner. These observations confirmed the importance of TLR3 activation in perturbing epidermal lipid levels, but suggest that other

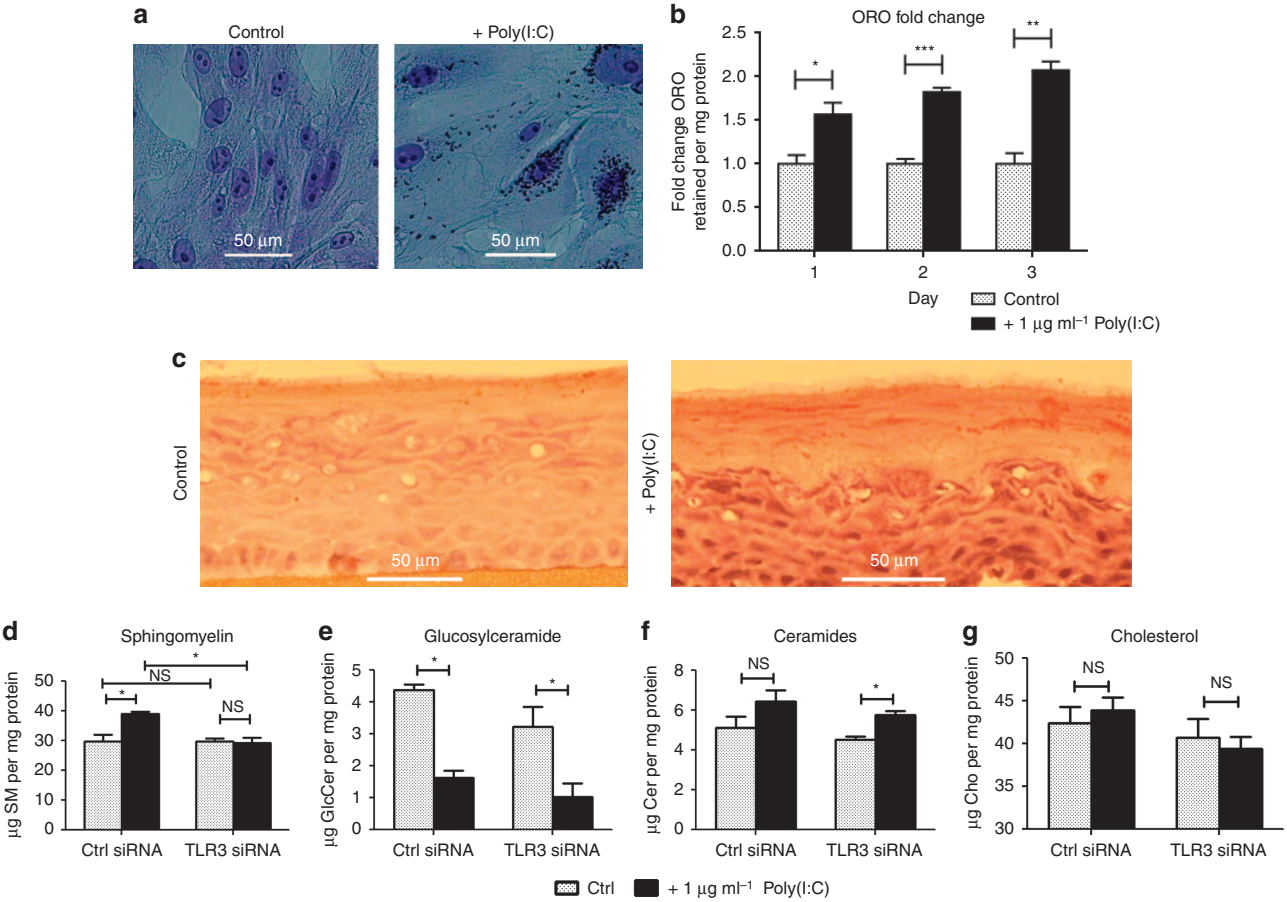


Figure 4. Activation of Toll-like receptor 3 (TLR3) alters epidermal lipid content. (**a**) Normal human epidermal keratinocytes (NHEKs) were treated for 72 hours with 10 μg ml^{-1} polyinosinic acid:polycytidylic acid (Poly (I:C)), stained with Oil Red O (ORO) and counterstained with hematoxylin. Bar = 50 μm. (**b**) NHEKs were treated with 1 μg ml^{-1} Poly(I:C) for 1, 2, or 3 days, and then stained with ORO. (**c**) The three-dimensional (3D) tissue constructs were treated with 1 μg ml^{-1} Poly(I:C) for 72 hours. Samples were stained with ORO and counterstained with hematoxylin. Scale bar = 50 μm. (**d–g**) NHEKs were treated with 1 μg ml^{-1} Poly(I:C) for 24 hours following small interfering (siRNA) knockdown of TLR3. (**d**) Sphingomyelin, (**e**) glucosylceramide, (**f**) ceramides, and (**g**) cholesterol were quantified using high-performance thin-layer chromatography (HPTLC) relative to total protein. *P<0.05; **P<0.01; ***P<0.001. Two-tailed t-test. Data are mean ± SEM, n = 3, and are representative of at least three independent experiments.

pathways influenced by the addition of Poly(I:C) also contribute to the response of some lipids. It remains to be determined whether Poly(I:C) treatment of NHEKs can stimulate *de novo* lipid synthesis. As our mRNA data of a number of lipid metabolism genes show that dsRNA can alter these transcripts, it is possible that the enzyme activity could also be altered, but this remains to be explored. As Oil Red O–positive vesicles most likely contain a mix of nonpolar lipids, we do not believe that the TLR3-dependent increases in sphingomyelin are being detected in those experiments, rather other lipid species that have yet to be identified. Future studies will hopefully elucidate whether *de novo* lipid synthesis is occurring and which additional lipid species are increased in a TLR3-dependent manner.

To further investigate functional changes in keratinocytes following Poly(I:C) treatment, we examined ultrastructural changes in keratinocytes within 3D skin constructs. Treatment of keratinocytes with Poly(I:C) yielded a higher amount of both LBs and KHGs in the granular layer of the epidermis. Although LBs have been found to be depleted in the granular layer following barrier disruption due to their rapid trafficking and secretion of barrier components (Menon *et al.*, 1992b), they are rapidly regenerated to aid in future barrier repair as well as proper differentiation and barrier formation. We speculate that TLR3 activation by endogenous dsRNA could be an initiation event that leads to downstream effects of epidermal lipid production, trafficking, and metabolism. Increases in KHGs also provide further evidence that Poly(I:C) treatment could promote barrier formation or repair as KHGs also contain essential barrier components of the SC including profilaggrin and LOR. Though we do not see increases in transcripts of FLG and LOR after Poly(I:C) treatment, the increased presence of KHGs provides evidence suggesting that dsRNA can influence barrier formation.

We believe that this work identifies a key recognition event that could trigger some elements of skin barrier formation during the process of repair. How this recognition event is propagated after TLR3 activation remains to be determined. However, these results could be utilized in the treatment of certain dermatological diseases such as atopic dermatitis (AD).

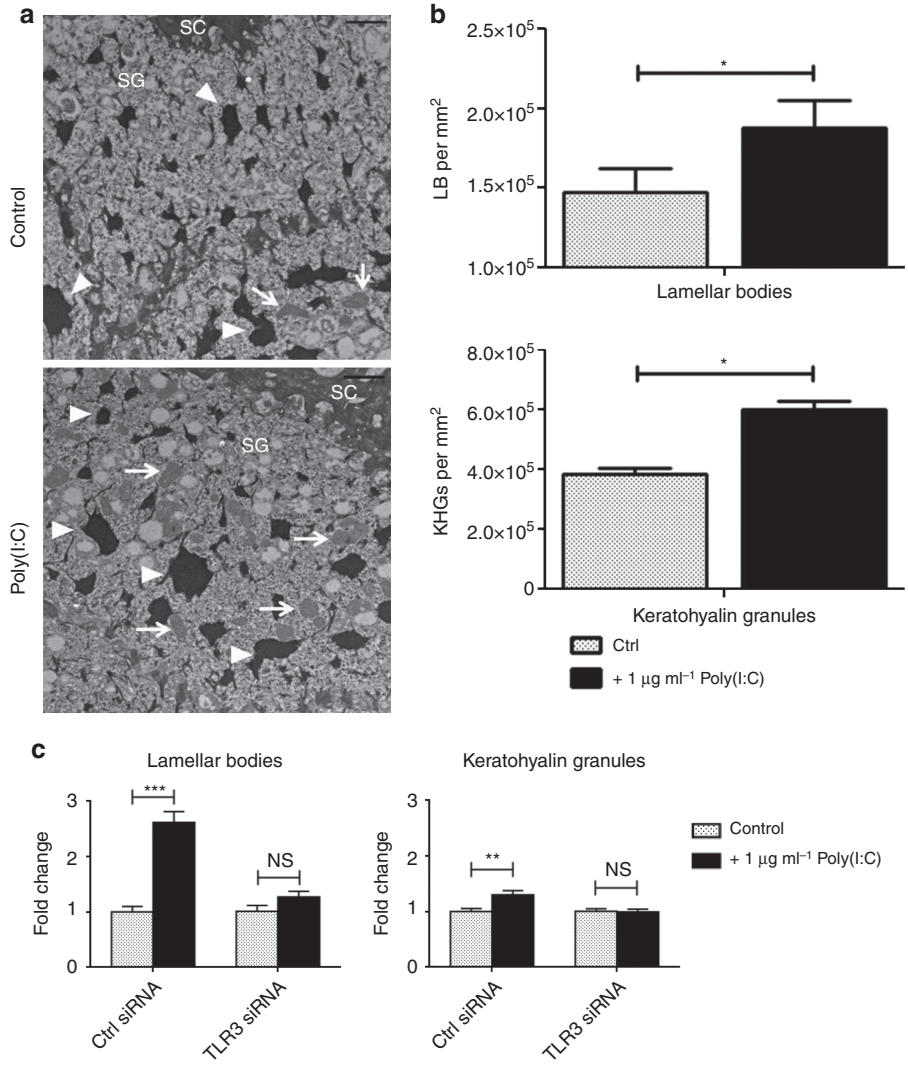


Figure 5. Toll-like receptor 3 (TLR3) activation increases the quantity of lamellar bodies (LBs) and keratohyalin granules (KHGs) in the epidermis. (**a**) Transmission electron microscopy of three-dimensional (3D) skin construct treated with 1 μg ml^{-1} polyinosinic acid:polycytidylic acid (Poly(I:C)) for 72 hours. Arrows indicate LBs and arrowheads indicate KHGs. Bar = 1 μm. SC, stratum corneum; SG, stratum granulosum. (**b**) Quantification of LBs and KHGs. *P<0.05; one-tailed t-test. Control (Ctrl; n = 54), 1 μg ml^{-1} Poly(I:C) (n = 47). Data are mean ± SEM. (**c**) Quantification of LBs and KHGs in TLR3 knockdown normal human epidermal keratinocytes (NHEKs). *P<0.05, **P<0.01, ***P<0.001, NS, nonsignificant. One-tailed t-test. Control small interfering RNA (siRNA) control (n = 54), control siRNA + 1 μg ml^{-1} Poly(I:C) (n = 51), TLR3 siRNA control (n = 55), TLR3 siRNA + 1 μg ml^{-1} Poly(I:C) (n = 54). Data are mean ± SEM.

For years, it has been known that AD patients have significantly decreased ceramide levels in the SC (Melnik *et al.*, 1988; Yamamoto *et al.*, 1991). Although some recent studies have shown AD to be associated with *FLG* mutations (Palmer *et al.*, 2006), a number of reports cite and characterize cases of AD that are independent of *FLG* mutations (Jasaka *et al.*, 2011) and demonstrate that AD patients have abnormal ceramide profiles and lamellar lipid organization (Ishikawa *et al.*, 2010; Janssens *et al.*, 2011). From our research, it could be speculated that this deficiency of ceramides in the SC of AD patients could be treated by pharmacological activation of pathways downstream of TLR3, thus leading to increases in ceramides. Of course, many unwanted inflammatory side effects may result from TLR3 activation, and hence it will be important to determine specifically which pathways downstream of TLR3 are involved in the increase of epidermal lipids. By examining these downstream pathways, we may also discover more about the regulatory events involved in ceramide biosynthesis and metabolism that could be affected in AD patients. Future studies will involve identifying and characterizing these downstream pathways of TLR3 activation relevant to barrier repair.

MATERIALS AND METHODS

Cell culture and stimuli

NHEKs were obtained from Cascade Biologics/Invitrogen (catalog number: C-001-5C; Portland, OR), and grown in serum-free EpiLife cell culture media (Cascade Biologics/Invitrogen) containing 0.06 mM

Ca^{2+} and 1 × EpiLife Defined Growth Supplement (EDGS, Cascade Biologics/Invitrogen) at 37 °C under standard tissue culture conditions. All cultures were maintained for up to eight passages in this medium with the addition of 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, and 250 ng ml^{-1} amphotericin B. Cells at 60–80% confluence were treated with Bafilomycin A1 (5 nM; Sigma, St Louis, MO), poly(I:C) (1 μg ml^{-1}; Invivogen, San Diego, CA), IL-6 (10 ng ml^{-1}; R&D Systems, Minneapolis, MN), or TNF-α (50 ng ml^{-1}; Chemicon, Temecula, CA) in 12-well flat-bottom plates (Corning Incorporated Life Sciences, Lowell, MA) for up to 24 hours. After cell stimulation, RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA). RNA was stored at − 80 °C.

For visualization or analysis of lipids, cells were switched to advanced-stage differentiation media (DMEM/F-12 (2:1), 10% fetal bovine serum (Invitrogen), 400 ng ml^{-1} hydrocortisone (Sigma), 10 μg ml^{-1} human recombinant insulin (Sigma), and freshly made 50 μg ml^{-1} ascorbic acid (Sigma)) for 24 to 72 hours during stimulation. Media was changed every other day and prepared with freshly dissolved, filter-sterilized vitamin C.

Quantitative real-time PCR

Total RNA was extracted from cultured keratinocytes using TRIzol Reagent (Invitrogen) and 1 μg RNA was reverse-transcribed using iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA). Pre-developed Taqman Gene Expression Assays (Applied Biosystems, Foster City, CA) were used to evaluate mRNA transcript levels of ABCA12, GBA, SMPD1, TGM1, TNF, IL-6, KRT1, KRT14, involucrin, FLG, LOR, and TLR3. Glyceraldehyde-3-phosphate dehydrogenase mRNA transcript levels were evaluated using a VIC-CATCCATGACAACTTTGGTA-MGB probe with primers 5′-CTTAGCACCCCTGGCCAAG-3′ and 5′-TGGTCATGAGTCCTTCCACG-3′. All analyses were performed in triplicate and were representative of three to five independent cell stimulation experiments that were analyzed in an ABI Prism 7000 Sequence Detection System (Life Technologies, Carlsbad, CA). Fold induction relative to glyceraldehyde-3-phosphate dehydrogenase was calculated using the $\Delta\Delta C_t$ method. Results were considered to be significant if $P<0.05$.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

We thank J Sprague, G Hardiman, and R Sasik at the UCSD Biomedical Genomics laboratory for assisting in microarray analysis. We thank Y Jones and T Meerloo at the UCSD EM Core for processing samples and helpful discussion and Dr Marilyn Farquhar for helpful discussion of EM images. This work was supported by US National Institutes of Health (NIH) grants R01-AR052728, R01-AI052453, and R01-AI0833358 to RLG, the UCSD Training in Immunology grant 5T32AI060536-05, and UCSD Dermatologist Investigator Training Program grant 1T32AR062496-01 supporting AWB.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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